The Incidence of Both Tandem Duplications and the Common Deletion in mtDNA from Three Distinct Categories of Sun-Exposed Human Skin and in Prolonged Culture of Fibroblasts

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The use of mtDNA damage as a biomarker of cumulative sunlight exposure in human skin is a relatively new field of research. Previous investigations have simply compared the frequency of occurrence of the mtDNA common deletion (CD), and to a much lesser extent that of tandem duplications (TDs), to distinguish between sun-protected and sun-exposed skin. This approach is limited because non-melanoma skin cancer is predominantly formed on body sites that are "usually" sun-exposed as opposed to sites that are "occasionally" sun-exposed and as such they differ in their cumulative UV exposure. This study addresses this limitation by investigating the frequency of occurrence of the CD and TDs in 116 age-matched human skin samples taken from three different sun-exposed body sites. There was a greater frequency of the mtDNA damage in "usually" sun-exposed compared to "occasionally" sun-exposed body sites for both the CD and the TDs ($P<0.0001$ and $P=0.058$, respectively). In addition, we identified a 260 bp triplication of the mtDNA D-loop for the first time in skin. No evidence of the CD or TDs was observed in sun-protected (ie rarely exposed) skin ($n=20$). Comparatively little is known about mtDNA damage in prolonged skin cell culture. We have furthered this work by studying the level of the CD and the frequency of the TDs during continued culture of human fibroblasts derived from skin samples taken from usually sun-exposed sites ($n=7$ patients). The level of the CD decreases with culture, whereas the frequency of TDs can be maintained.

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INTRODUCTION

Human mtDNA is a 16,569 bp circular, double-stranded DNA molecule present in approximately 2–10 copies per mitochondrion. Mitochondria are responsible for producing approximately 90% of the cells energy in the process of oxidative phosphorylation. The mtDNA genome codes for 13 polypeptides that are all components of the respiratory chain along with two rRNAs and 22 tRNAs that are responsible for expression of these polypeptides.

mtDNA deletions are readily detected in non-dividing tissues such as brain and muscle, but are rarely detected in cells with a relatively short half-life (eg blood cells). The most frequently reported deletion is a 4,977 bp deletion or ''common deletion'' (CD), which was originally observed in patients with mitochondrial myopathies (Wallace, 1992). The CD has been extensively studied and has shown an age-related accumulation in various tissues including brain, heart, kidney, muscle, liver, and blood (Corral-Debrinski *et al.*, 1992; Cortopassi *et al.*, 1992; Simonetti *et al.*, 1992; Kadenbach *et al.*, 1993; Melov *et al.*, 1995). The CD has also been shown to occur in higher frequency with increasing sun exposure, with higher levels of the deletion (>1%) being associated with photo- rather than chronological ageing (Pang *et al.*, 1994; Yang *et al.*, 1994, 1995; Berneburg *et al.*, 1997; Birch-Machin *et al.*, 1998; Birch-Machin, 2000).

The D-loop is the key regulatory site of the mtDNA genome. It contains the origin of H strand replication and contains the promoters for transcription of both strands. A 260 bp tandem duplication (TD) has been reported in the D-loop and was proposed to be associated with large-scale deletions such as the CD (Brockington *et al.*, 1993). Following this report, TDs of 150 and 200 bp in size and the previously reported 260 bp TD have been found to occur in an age-dependent manner in tissues such as muscle, testis, and skin (Lee *et al.*, 1994a; Wei *et al.*, 1996). These TDs have also been reported to occur at a higher frequency in sun-exposed skin than in non-sun-exposed skin (Yang *et al.*, 2004).

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Abbreviations: CD, common deletion; TD, tandem duplication

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UVR is the major determinant of non-melanoma skin cancer. It is an important factor in the generation of oxidative damage, which can lead to DNA strand breaks and to the production of DNA photoproducts such as thymidine dimers, which cannot be repaired in mtDNA (Clayton *et al.*, 1974). In addition, mtDNA is located in the matrix, which is in close proximity to the inner membrane where reactive oxygen species are continually produced in the electron transport chain. This along with the absence of protective histones makes mtDNA a sensitive marker of UV-induced DNA damage compared to nuclear DNA (LeDoux *et al.*, 1992; Croteau and Bohr, 1997; Pascucci *et al.*, 1997; Sawyer and Van Houten, 1999; Birch-Machin, 2000). Finally, each cell can contain up to several thousand copies of the mtDNA genome, and mitochondria can therefore tolerate very high levels (up to 90%) of damaged mtDNA through complementation of the remaining wild type (Chomyn *et al.*, 1992; Sciacco *et al.*, 1994). Therefore, cells are able to accumulate photodamage in mtDNA without compromising cell function.

Recently, we found that a 3,895 bp mtDNA deletion occurred more frequently in usually sun-exposed skin as opposed to occasionally sun-exposed skin (Krishnan *et al.*, 2004). Limitations of other studies, including our own, looking at the incidence of the CD (Yang *et al.*, 1994, 2004; Berneburg *et al.*, 1997; Birch-Machin *et al.*, 1998), have grouped together skin sites from varying areas of sun exposure. This could potentially mask results from usually sun-exposed areas (eg face and hands) compared to occasionally sun-exposed areas (eg trunk and legs). In the present study, we have investigated the frequency of occurrence of the CD in split skin samples taken from three separate areas of sun exposure when outdoors as defined previously by Armstrong (2004), namely rarely, occasionally, and usually sun exposed. Owing to the previous postulated associations between the CD and TDs, the frequency of occurrence of TDs and its relationship to the CD was also investigated. The TD work in the current literature has been derived predominantly from a single laboratory, which has reported a further nine different TDs in addition to the original 260 bp TD observed by Brockington *et al.* (Wei *et al.*, 1996). The majority of these novel TDs however have not yet been confirmed by another independent laboratory. Furthermore, these studies have looked at the incidence of the TDs in whole skin samples, yet previous studies by our group and confirmed by others (Birch-Machin *et al.*, 1998; Ray *et al.*, 2000) have used split skin samples to show that mtDNA damage (eg the CD) predominates in the dermis compared with the epidermis. The present study has therefore investigated the frequency of occurrence of TDs in both epidermis and dermis.

It has been shown that the CD can be induced in human dermal fibroblasts by repetitive doses of UVA (Berneburg *et al.*, 1999). Another study by Koch *et al.* (2001) described how UVR increased the level of the CD in human keratinocytes. They also showed that the levels of the CD decreased after subsequent culture, compared with immediate harvesting of the cells. In the present study, we have furthered this work by looking at the incidence of the CD in human fibroblasts derived from skin samples taken from usually sun-exposed skin sites of aged patients. In addition, we have determined whether there is a relationship between the CD and TDs, both in skin and in the derived cultured skin fibroblasts.

RESULTS

A higher frequency of occurrence of the common deletion in usually *versus* occasionally sun-exposed skin

We have previously shown that the presence of a 3,895 bp deletion of mtDNA is able to distinguish between usually and occasionally sun-exposed sites (Krishnan *et al.*, 2004). This is significant, as the majority of non-melanoma skin cancers are formed on areas of usually sun-exposed skin. Limitations to previous studies looking at levels of the CD in human skin including our own (Wei *et al.*, 1996; Berneburg *et al.*, 1997; Birch-Machin *et al.*, 1998) have failed to address the difference in cumulative UVR exposure in these two distinct categories of sun exposure when outdoors.

A quantitative, radioactive three-primer PCR analysis was performed on 116 age-matched, split skin samples from different body sites to compare the frequency and levels of the CD. The samples were divided into three categories depending on their sun exposure as defined by Armstrong (2004), namely usually sun exposed (eg face and hands), occasionally sun exposed (eg trunk and legs), and rarely sun exposed (eg buttock and heel). Low levels of the CD, typically much less than 0.2%, have been associated with increasing age (Nagley and Wei, 1998). Therefore, to ensure that the effects of ageing did not confound our results, positive results for the CD were counted as levels above 1%. The results showed that there is a significantly higher ($P<0.0001$, Fisher's exact test) frequency of occurrence of the CD in usually sun-exposed samples (55%, $n=28/51$), compared with those seen in the occasionally sun-exposed samples (2%, $n=1/45$) (Figure 1). The CD was absent in the 20 rarely sun-exposed skin samples. In those usually sun-exposed sites where the CD was present, the levels of the CD were typically between 1 and 25%.

Further investigation of the usually sun-exposed samples showed that the dermis harbored a higher frequency of the

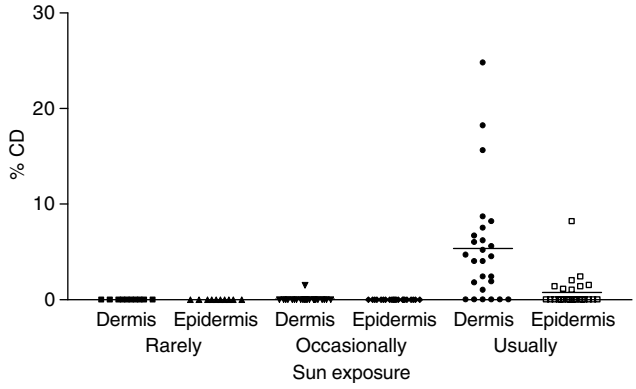


Figure 1. The level of the CD expressed as a ratio to wild-type mtDNA is increased with the increasing sun exposure of body sites when outdoors.

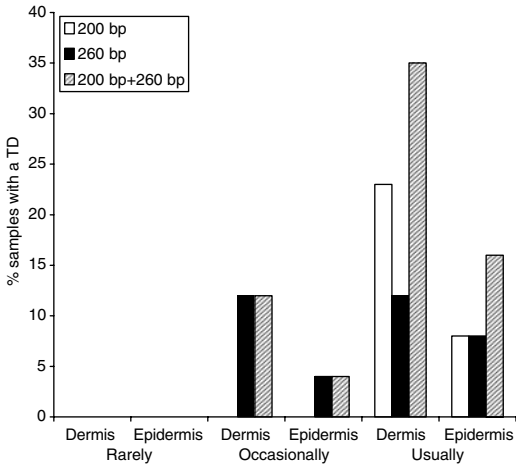


Figure 2. The frequency of occurrence of TDs increases with increasing sun exposure of body sites when outdoors.

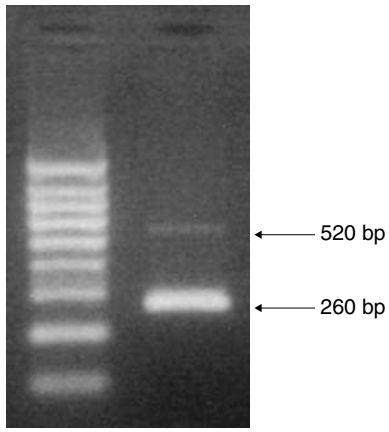


Figure 3. A typical agarose gel following PCR amplification with primers L336 and H335 to show the presence of a 260 bp TD as well as a 520 bp triplication.

CD than the corresponding epidermis. In detail, the frequency of occurrence of the CD within the dermis is 77% ($n=20/26$), which is significantly higher ($P=0.002$, Fisher's exact test) than that found in the corresponding epidermal samples where 32% ($n=8/25$) of samples harbored the CD. Incidentally, the only occasionally sun-exposed sample to harbor the CD was also a dermis sample (Figure 1).

A higher frequency of occurrence of the 200 and 260 bp tandem duplications in usually *versus* occasionally sun-exposed skin

The same 116 age-matched, split skin samples from differing sites of sun exposure were screened to assess the incidence of the 200 and 260 bp TDs using a non-quantitative ''back to back'' primer methodology. The presence of the duplications was confirmed by automated DNA sequencing. The mtDNA sequences contained repeat breakpoints at np (nucleotide position) 5′567/CCCCCC/308 3′ and 5′ 493/CCCCC/307 3′, confirming the presence of the 260 and 200 bp TDs, respectively. The results showed an increase in the frequency of occurrence of the TDs with increasing sun exposure (Figure 2). The TDs were present in 25% ($n=13/51$) of usually sun-exposed samples compared with 9% ($n=4/45$) of the occasionally sun-exposed samples. This difference between sun-exposure sites was not statistically significant ($P=0.058$, Fisher's exact test). None of the samples from rarely sun-exposed skin sites was found to harbor a TD.

There was a higher frequency of TDs in the dermis than the epidermis, an observation that is similar to the pattern of the CD in the same skin samples. For example, in usually sun-exposed skin the frequency of occurrence of the TDs was 35% in the dermis (9/26) and 16% in the epidermis (4/25), and in occasionally sun-exposed skin the frequency of occurrence of the CD was 13% in the dermis (3/23) and 5% in the epidermis (1/22). However, this difference in both the usually and occasionally sun-exposed skin was not significant (Fisher's exact test).

The identification of a 260 bp triplication in human skin

While performing the back to back PCR assay using primers L336/H335, there was a skin sample whose DNA produced a faint band of 520 bp, which was in addition to the 260 bp TD (Figure 3). Sequencing analysis of the 520 bp PCR band revealed it to be a 260 bp triplication. This is the first time a 260 bp triplication has been identified in human skin, as it has previously been reported only once before (Moraes *et al.*, 1992) in a patient with a mtDNA disorder. Further analysis of our samples showed that the 260 bp triplication was seen in two dermis samples, both from usually sun-exposed skin, and both the samples also contained a 260 bp duplication.

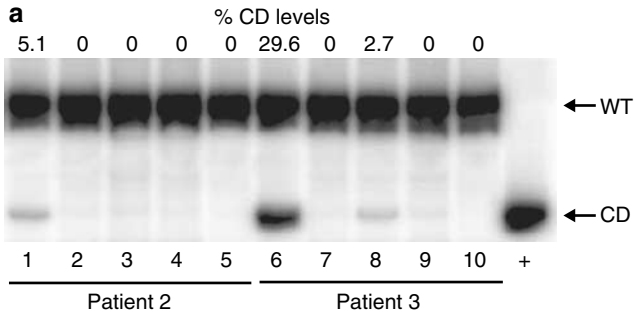
The identification of other tandem duplication species in the D-loop region

A single study by Wei *et al.* (1996) reported the identity of a further eight TDs in the D-loop region of human skin besides the previously reported 200 and 260 bp TDs. Only one other group has confirmed the presence of one of these other TD species in muscle from a patient with a mitochondrial disorder (Bouzidi *et al.*, 1998). Our study investigated the incidence of these additional TD species in the D-loop using different sets of ''back to back'' primer pairs (Table 1) as described by Wei *et al.* (1996) in 20 split skin samples from both usually and occasionally sun-exposed skin. These investigations identified only two of the additional TD species. One of these was a 650 bp TD and its identity was confirmed by sequencing. This duplication was found in only one sample from the dermis of a usually sun-exposed skin sample. Wei *et al.* (1996) have previously proposed that there are two types of 200 bp TDs, type II (previously identified) and type IX, which are distinct, and both can be identified using primer pairs H446 and L467. Our study identified this other type of 200 bp TD (type IX) in a dermis sample from usually sun-exposed skin. As both types of 200 bp TDs can be amplified using primers H466 and L467, it is difficult to readily determine the frequency of each type of 200 bp TD.

Table 1. Oligonucleotide primer pairs used to detect the different types of TDs reported by Wei *et al.*, and the corresponding duplication size

Primer pairs	Type of TD	Size of TD (bp)
L336–H335	I	260
	II	200
	III	150
L616–H599	IV	650
L258–H16509	V	560
	VI	500
	VII	500
	X	500
L258–H16450	V	370
	VI	310
	VII	310
	X	310
L336–H16450	VI	420
	VII	420
	VIII	380
	X	420
L467–H466	I	260
	II	200
	IX	200
L501–H466	IX	170

L258 np 258–277; L336 np 336–355; L467 np 467–486; L501 np 501–520; L616 np 616–635; H335 np 335–316; H466 np 466–447; H599 np 599–580; H16450 np 16,450–16,431; H16509 np 16,509–16,490.



b

	% level of CD (n=3)				
Patient	Dermis	Epidermis	Passage 3	Passage 7	Passage 12
1	6.2	≤1	≤1	≤1	≤1
2	**5.6**	**≤1**	**≤1**	**≤1**	**≤1**
3	**24.8**	**≤1**	**3**	**≤1**	**≤1**
4	≤1	≤1	≤1	≤1	≤1
5	≤1	≤1	≤1	≤1	≤1
6	17.6	≤1	≤1	≤1	≤1
7	20.4	≤1	≤1	≤1	≤1

Figure 4. (a) A representative three-primer PCR gel showing the levels of the CD in dermis, epidermis, and fibroblasts from two patients. Lanes 1 and 6 are dermis samples; lanes 2 and 7 are epidermis samples; lanes 3 and 8 are fibroblasts at passage 3; lanes 4 and 9 are fibroblasts at passage 7; lanes 5 and 10 are fibroblast lines at passage 12. + = positive control. Above the gel are the % CD levels for each lane as calculated by ImageQuant software. The PCR was repeated three times to ensure accurate quantification of the % CD levels. (**b**) Percentage CD levels for each of the seven fibroblast lines at three different passages, as calculated by ImageQuant software following three-primer PCR amplification. The average was calculated from three separate PCR reactions. <1% = not detected. In bold and highlighted are the two patient samples shown in (**a**).

The level of the common deletion decreases with culture in fibroblasts whereas the frequency of occurrence of the tandem duplication is maintained

Fibroblasts were cultured from the dermis of seven aged patients (mean age 73 years). The three-primer PCR was performed on split skin samples from the seven patients to determine the presence of the CD. Figure 4a shows an example of a typical three-primer PCR gel. For each sample, the percentage level of the CD (in comparison to wild-type mtDNA levels) was calculated using ImageQuant software. None of the epidermal samples was found to harbor the CD. DNA analysis of the dermal fibroblasts cultured from the seven patient samples revealed that only 1/7 fibroblast lines showed the presence of the CD. The level of the CD in this fibroblast line from patient 3 was quantified to be 3% (Figure 4b) and was only detectable at passage 3, as subsequent passages led to decreased levels of the CD to below 1%. DNA analysis of the remaining six fibroblast lines did not reveal the presence of the CD in any of the passages (Figure 4b). Interestingly, the fibroblast line in which the CD was detectable also had the highest level of CD in the corresponding dermis (24.8%) compared to all the other six patient samples that were studied (Figure 4b).

Using a ''back to back'' PCR methodology, the presence of the TDs in the D-loop was determined in the same split skin samples from the seven patients and the derived fibroblast cell lines. None of the epidermal samples contained a TD, but it was found that 2/7 dermal samples (patients 2 and 5) were found to harbor a TD of size 200 bp. Of these two patient samples, the TD was not detectable in the fibroblast lines derived from patient 5 (data not shown) but was observed and maintained up to passage 12 in the fibroblast line derived from patient 2. Interestingly, patient 2 harbored a higher level of the CD (CD = 5.6%) than patient 5, who did not harbor the TD in culture (CD = not detected) (Figure 4b). The absolute levels of the TD in both samples are currently unknown owing to a lack of existing methodology by which the level of the TDs can be determined.

DISCUSSION

The common deletion occurs with a higher frequency in samples from usually sun-exposed skin

Previous works by us and others have shown a UVR-associated increase in the incidence of the mtDNA damage in sun-exposed compared to rarely sun-exposed human skin when outdoors. The major limitation of these studies is their failure to address the fact that sun-exposed body sites are

divided into two distinct categories, usually (ie face and hands) and occasionally (ie trunk and legs) (Armstrong, 2004). This is vitally important for skin cancer research as the vast majority of non-melanoma skin cancers occur on usually sun-exposed sites.

The results obtained in the present study confirm previous findings that the frequency of the CD is increased with increasing sun exposure (Birch-Machin *et al.*, 1998). Our present study has extended this work because it was found that the frequency of the CD is significantly higher ($P<0.0001$, Fisher's exact test) in usually sun-exposed compared with occasionally sun-exposed body sites. Our study has also confirmed previous findings that the frequency and level of the CD is predominantly higher in the dermis than in the epidermis, but we have also extended the work by showing that this pattern is observed both in the occasionally sun-exposed samples and in the usually sun-exposed skin.

The CD deletes the genome between np 8,482 and 13,460, which includes the deletion of adenosine triphosphatase 8 (complex V) to ND5 gene encoding subunit 5 of complex I (complex I), and also involves the deletion of adenosine triphosphatase 6 (complex V), cytochrome *c* oxidase III (complex IV), ND3, ND4L, ND4 (complex I) as well as six tRNAs. The CD levels observed in skin in this study are between 1 and 25% and therefore may have a partial deficiency effect on oxidative phosphorylation. It is known that in human muscle, individual fibers can differ in the level of mtDNA deficiency throughout the muscle and that these differing levels will contribute to an overall average of mtDNA deficiency in a given muscle sample (Brierley *et al.*, 1998). It is therefore more likely that the levels observed in skin (ie 1–25%) represent an overall average and that individual cells and/or focal areas will have high (ie CD > 50%) areas of damage and low areas of damage throughout the sample. This could therefore result in oxidative phosphorylation deficiency in these focal areas. This is further complicated by the recessive nature of mtDNA (Chomyn *et al.*, 1992; Sciacco *et al.*, 1994), the threshold of mtDNA damage that is required to give a biochemical consequence (Porteous *et al.*, 1998), and the capacity of mtDNA to harbor a high mutation load. The effect of this can also be dependent on the energetic capacity of the tissue, as there is a higher proportion of mitochondria in tissues with high energy demands. The resultant effect in skin however is uncertain at present.

It has been previously suggested that the mechanism for the generation of the CD involves intragenomic recombination event via slipped strand mispairing and this may occur at the 13 bp repeats flanking the CD (Schon *et al.*, 1989; Shoffner *et al.*, 1989; Mita *et al.*, 1990; Degoul *et al.*, 1991). This mechanism requires regions of single-stranded DNA on both the heavy and light mtDNA strands, but neither of the 13 bp repeats are simultaneously single-stranded during replication (Schon *et al.*, 1989). Schon *et al.* also suggested that homopyrimidine stretches in the 13 bp repeat/or flanking AT-rich regions may be susceptible to DNA bending that would allow a small region or ''bubble'' of single-stranded DNA to open. Interestingly, these DNA stretches map to structurally labile ''hot regions'' for the CD that assume an unusual bent-DNA structure and that may therefore enhance the intragenomic recombination event (Hou and Wei, 1996). More recently, it has been observed that the distribution of the majority of mtDNA deletions with direct repeats (>4 bp) occurs around the 13 bp direct repeat of the CD (Samuels *et al.*, 2004). The authors of this study have proposed that factors behind the generation of the CD may also influence the formation of the majority of other mtDNA deletions. Our results suggest that UVR is a contributing factor in the generation of the CD. We propose that prolonged UVR exposure could either directly (by inducing base substitutions as opposed to deletions) or indirectly (by induction of free radicals) affect these structurally labile sites by opening a ''bubble'' of single-stranded DNA that would enhance the recombination event, thereby eliciting an increase in mtDNA deletions.

Tandem duplications of the D-loop occur with a higher frequency in samples from usually sun-exposed skin

The D-loop is the control region for both replication and transcription of human mtDNA. In this study, we have determined the incidence of TDs of the D-loop region in the same split skin samples used for the study of the CD, that is, taken from three different areas of sun exposure. In a similar fashion to the frequency of occurrence of the CD, the TDs also showed a higher frequency in usually *versus* occasionally sun-exposed body sites, albeit with lower abundance. In addition, the incidence of the TDs shows the same pattern as that for the CD, namely that the dermis harbors a higher proportion of TDs than the epidermis.

Interestingly, the 5′-ends of both the 200 and 260 bp TD occur at the poly(C) stretch starting from np 303–316 (5′-CCCCCCCTCCCCCC-3′), which is in close proximity to the conserved sequence block II, which is important for the regulation of the initiation of mtDNA replication. We have observed that the number of C's at this region can vary between individuals (data not shown). The significance of and mechanism causing the additional C nucleotides in the D-loop are unclear. It is therefore tempting to hypothesize that an increased number of C's at this region increases the likelihood of the development of a TD, through a mechanism of slip-strand mispairing at these hot spots containing poly(C) stretches (Shoffner *et al.*, 1989).

We investigated the incidence of a further eight reported TDs in addition to the original 200 and 260 bp species. These eight additional TDs have been previously reported in a single study by Wei *et al.* (1996). Furthermore, only one other laboratory has confirmed the existence of one of these eight additional TDs (Bouzidi *et al.*, 1998). Our investigations found only two of these eight proposed TDs in DNA samples taken from usually sun-exposed skin. Sequencing analysis revealed that these additional TDs are a 650 bp species and another 200 bp TD (type IX as described by Wei *et al.*, 1996) that is distinct from the original 200 bp TD. The repeat breakpoint of the 650 bp TD includes the run of C's at np 303–316; so the mechanism of generation, although unclear at present, may be similar to that proposed for the generation

of the original 200 and 260 bp TD. The repeat breakpoint of the additional 200 bp TD (type IX) includes a series of C's and A's at np 347–359 (5′-CCAAACCCCAAA-3′); so the mechanism of generation may occur through a different mechanism involving slip-strand mispairing combined with single-strand breaks (Bouzidi *et al.*, 1998).

It is still uncertain in the literature whether TDs in the D-loop are pathogenic to mtDNA (Brockington *et al.*, 1993; Hao *et al.*, 1997). It is thought that any type of mutations, particularly duplication of the regulatory elements in the D-loop of mtDNA, could potentially cause alterations of DNA replication and gene expression in the mitochondria.

The dermis harbors a higher proportion of mtDNA damage than the epidermis

The results of the present study and those reported by other studies (Birch-Machin *et al.*, 1998; Ray *et al.*, 2000) show that the dermis seems to harbor a higher level of mtDNA damage than the epidermis. At first, this observation would seem unexpected, as the epidermis, being the upper layer of the skin, receives a greater proportion of UVR and also acts as a filter of UVR for the dermis. However, based on previous studies in several tissues (Ikebe *et al.*, 1990; Corral-Debrinski *et al.*, 1991; Yen *et al.*, 1991; Lee *et al.*, 1994b), it has been proposed (Cortopassi *et al.*, 1992) that there is more CD in tissues that turn over slowly or not at all (eg brain and muscle) than in those that turn over relatively more rapidly (eg liver and blood cells). This effect may be explained by the fact that if mtDNA mutations favor an imbalance in the composition of the respiratory chain, and if such an imbalance enhances reactive oxygen species production and oxidative damage, mitochondria harboring mutant mtDNA will in fact suffer a disadvantage during the energy-consuming process of replication (Kowald, 1999). This effect will be highest in rapidly dividing cells. Therefore, according to this proposal, the high turnover of keratinocytes may result in the accumulation of mtDNA deletions in little time, whereas in contrast the low proliferative rate of fibroblasts may result in the accumulation of deleted mtDNA. Furthermore, the lower mutational load in the epidermis would be enhanced by the much higher activity of the antioxidant enzymes superoxide dismutase and catalase in the epidermis than in the dermis (Shindo *et al.*, 1994).

The common deletion is decreased with prolonged culture of dermal fibroblasts

Prolonged culture of fibroblasts derived from seven skin biopsies was performed to determine the frequency of occurrence of the CD during proliferation. As the deletion does not incorporate the two replication starting points of mtDNA (O_H and O_L), deleted mtDNA molecules were thought to show wild-type or even higher replication rates owing to their reduced length (Clayton, 1982). In contrast, in the present study, we observed a decrease in the relative amount of CD with prolonged culture. These results agree with the observations by Koch *et al.* (2001) where the level of CD in cultured adult keratinocytes decreased by 90% after two passages. Interestingly however, the levels observed by Koch *et al.* never exceeded 1%, whereas the level of CD in our fibroblast line is comparatively very high at ~3%. A possible mechanism for the disappearance of the CD with culture observed in the present study might be that replication is either reduced or blocked in deleted mtDNA genomes to allow degradation of mitochondria bearing the CD (Koch *et al.*, 2001).

A 200 bp tandem duplication can be maintained during prolonged culture of dermal fibroblasts

In contrast to those observations involving the CD, our present study shows that a 200 bp TD can be maintained during prolonged culture of a fibroblast line at least up to passage 12. The mechanism for this accumulation is unclear and we also identifed another patient in whom the 200 bp TD was present in the dermis but could not be detected in the derived fibroblast culture. In both cases, the skin was taken from a usually sun-exposed site. Interestingly, the patient sample that gave rise to the TD in culture also harbored a higher level of the CD (CD = 5.6%) than the patient sample that did not harbor the TD in culture and where the CD could not be detected. This brings into question a possible relationship between the occurrence of the CD and the 200 bp TD (Brockington *et al.*, 1993; Poulton *et al.*, 1993). However in our study, although the frequency of occurrence of the CD and TDs increases with UVR exposure, there appears to be no clear relationship between them.

SUMMARY

In summary, we have shown that the frequencies of the CD and TDs of the D-loop are significantly different between ''usually'' and ''occasionally'' sun-exposed body sites. In addition, although both the CD and TDs occur more frequently in areas exposed to sunlight, we found no clear evidence of a relationship between the two types of mtDNA damage. The frequency of damage to mtDNA such as the CD, TDs, and the previously reported 3895 bp deletion (Krishnan *et al.*, 2004) in human skin provides a potential biomarker for cumulative UV exposure. In this respect, our observation that TDs can be maintained in dividing skin cells may offer a powerful tool.

MATERIALS AND METHODS

Preparation of skin samples

Usually sun-exposed skin (face and hands) (epidermis $n = 25$, dermis $n = 26$, mean age = 68 years) and occasionally sun-exposed skin (trunk and legs) (epidermis $n = 22$, dermis $n = 23$, mean age = 67 years) were taken after obtaining written informed consent from 42 non-melanoma skin cancer patients with clinically normal perilesional skin, who attended the skin cancer excision clinic at the Royal Victoria Infirmary, Newcastle, UK. The proportion of male:female patients was similar for both the occasionally sun-exposed (50:50) and usually sun-exposed sites (60:40). Rarely sun-exposed skin (buttock and heel) was taken from previously obtained post-mortem samples (epidermis $n = 10$, dermis $n = 10$, mean

age = 76 years) (Krishnan *et al.*, 2004). The medical ethical committee of the University of Newcastle approved all the described studies. In addition, the study was conducted according to the Declaration of Helsinki Principles. None of the patients used for this study had a mtDNA defect. The epidermis and dermis were separated using 0.25% dispase at 4°C overnight (Jonason *et al.*, 1996). DNA from the skin samples was extracted using a DNeasy tissue extraction kit (Qiagen UK Ltd, Crawley, Sussex).

Culture of dermal fibroblasts

Fibroblasts were cultured from the dermis of normal skin taken from usually sun-exposed body sites (eg face and hands) of aged patients (mean age = 73 years). Separated dermis was cut into small pieces and placed in a minimal amount (to prevent the tissue from floating) of fetal calf serum overnight. After 24 hours, 5 ml of DMEM was added and placed in the incubator. After 5–7 days, fibroblasts had migrated out of the tissue, which were then removed. Dermal fibroblasts were cultured in T75 flasks containing 15 ml DMEM and 10% fetal calf serum. The fibroblasts were grown and passaged every 1–2 weeks. After passage 8, the cells became slow growing, were not dividing, and hence were not cultured beyond passage 12. DNA was extracted from fibroblasts at appropriate passages. DNA was also extracted from the corresponding split skin (ie dermis and epidermis).

Three-primer PCR to determine the level of the common deletion

The three-primer PCR was used, as previously described (Durham *et al.*, 2003). The PCR uses three primers, A, B, and C, where primer A resides within the CD region. In wild-type DNA, the distance between primers A and B is too large to amplify and so only one band is produced at 755 bp from primers A and C. Consequently, DNA containing the CD can amplify the region between primers A and B, so a second band is observed at 470 bp. The PCR reaction uses 100 ng/ml of target DNA in each reaction. Reaction was in a 25 μl volume of 200 μM dNTPs, NH_4 buffer (containing 160 mM $(NH_4)_2SO_4$, 67 mM Tris, pH 8.8) (Biotaq, Bioline, London, UK), 1.5 mM $MgCl_2$, 2.5 U/reaction *Taq* DNA polymerase, 25 pmol/μl of primers A and B, 6.25 pmol/μl primer C, and 3 μCi of [α-^{32}P]dCTP. The PCR conditions were 94°C for 4 minutes, 25 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 2 minutes, and a final extension of 8 minutes at 72°C. The PCR products were then electrophoresed through a 6% non-denaturing polyacrylamide gel and exposed to a phosphorimage film for approximately 24 hours. The radioactive PCR fragments were scanned and visualized by a PhosphorImager, using the ImageQuant software (Molecular Dynamics Ltd, Chesham, Bucks, UK).

Tandem duplication PCR ("back to back" primer methodology)

The desired mtDNA fragment was amplified in a 25 μl reaction containing 200 μM dNTPs, 1 U of HotStarTaq DNA polymerase (Qiagen), 0.4 μM of each primer, and 10 mM 10 × Buffer (Qiagen). See Table 1 for primers used. The DNA amplification reactions were carried out for 30 cycles in a Perkin-Elmer/Cetus DNA thermal cycler. The denaturation step was carried out at 94°C for 4 minutes, 30 cycles of 94°C for 1 minute, 55–57°C for 1 minute, 72°C for 1 minute, and a final extension of 72°C for 7 minutes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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